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(25) Most relevant to the present topic is the earlier observation that cyclopropylcarbinyl cations having 60° geometry appear considerably less stable than their counterparts with 0–30° geometries.[18c]
(26) The conformational features of the trishomocycloheptatriene hydrocarbon parent of **33** have been previously assessed.[17]

Sulfoxylate Ion (HSO_2^-), the Hydride Donor in Dithionite-Dependent Reduction of NAD^+ Analogues

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Abstract: At high pH interaction of dithionite with NAD^+ analogues results in formation of a sulfinate adduct. Its rate of formation is linearly dependent on dithionite concentration. Hence, sulfinate radicals do not appear to be involved in this process. A linear free energy relationship for adduct formation is obtained, the rate of which increases with increasing redox potential of the NAD^+ analogue. The deprotonated adducts are found to be very stable both thermodynamically ($K_d < 10^{-7}$ M) and kinetically ($k_{off} < 10^{-4}$ s^{-1}). Formation of NADH analogues is therefore not observed at pH >11. Conversion of adducts, formed from stoichiometric amounts of NAD^+ analogue and dithionite at high pH, to NADH analogues can be studied by pH jump, stopped-flow spectrophotometry: (1) After protonation of the sulfinate function, formation of oxidized NAD^+ analogue is observed in a fast initial phase (k for NAD^+ = 4.62 s^{-1}), the rate of which increases with decreasing redox potential of nicotinamide. (2) In a much slower, second phase, formation of NADH analogue is observed, which takes more than 20 min to completion. NADH formation can be prevented by adding formaldehyde, which traps the active reducing species. (3) If NAD sulfinate is mixed at pH 5 with an equimolar amount of the high-potential analogue 3-acetylpyridine-NAD^+ almost quantitative formation of 3-acetylpyridine-NADH is observed with no detectable formation of NADH. These results lead us to propose that sulfoxylate ion (HSO_2^-), a hydride donor formed after heterolytic dissociation of the protonated sulfinate adduct, is the active reducing species. Neither the sulfinate adduct itself nor sulfinate radicals appear to be productive in NADH formation. Hence, dithionite appears to be a selective, ambivalent reducing agent. While flavins are reduced by the homolytic dissociation product, sulfinate radical, nicotinamides are reduced by the heterolytic dissociation product, sulfoxylate ion. The factors controlling the nicotinamide pathway are both the high thermodynamic instability of the nicotinamide radical and the high stability of the sulfinate adduct.

Introduction

The question of $1e^-$ vs. $2e^-$ transfer in both flavin- and nicotinamide-dependent oxidoreduction is a currently disputed matter.[2-5] In particular, the possibility of sequential $1e^-$ transfer involving caged radical pair intermediates has been emphasized as a means of avoiding the high energy of the free nicotinamide radical.[2b,3,4,6] While electrochemical and pulse radiolysis studies are important for generating and characterizing free radicals, they suffer from the disadvantage that $1e^-$ transfer becomes mandatory when it might well be irrelevant under biologically meaningful conditions. Clearly, it is desirable to study reactants with readily available $1e^-$ and $2e^-$ shuttles because only then is a choice for a given pathway possible.

Dithionite is such a reactant which in fact has long been used as a general reducing agent in biochemical systems. Only very recently, however, Mayhew has shown that its $2e^-$ shuttle for the reaction

$$S_2O_4^{2-} + 2H_2O \rightleftharpoons 2HSO_3^- + 2H^+ + 2e^- \quad (1)$$

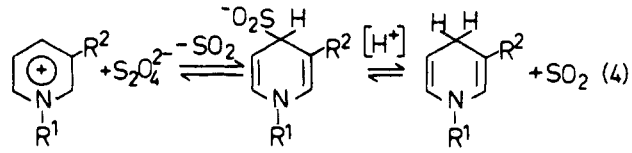
is characterized by an $E_0' = -386$ mV at 2 M sulfite[7] while the $1e^-$ shuttle derived from dissociation of dithionite into two sulfinate radicals

$$S_2O_4^{2-} \rightleftharpoons 2\dot{S}O_2^- \qquad K_d = 10^{-8}\ M \quad (2)$$

$$\dot{S}O_2^- + H_2O \rightleftharpoons HSO_3^- + H^+ + e^- \quad (3)$$

is characterized by an $E_0' = -660$ mV at concentrations smaller than K_d. At higher concentrations of dithionite E_0' increases by 29 mV for every tenfold increase in dithionite.[7] In the concentration range where it is usually applied (up to 10 mM) dithionite is thus both a strong $1e^-$ and $2e^-$ reductant.

Free flavins and flavodoxin are reduced by dithionite via consecutive $1e^-$ steps[8,9] and no intermediates have been observed. Nicotinamides, however, form sulfinate adducts, presumably via nicotinamide-C(4), which are stable at high pH but can be converted to 1,4-dihydronicotinamides by protonation of the sulfinate function (eq 4).[10-13] Although this reaction has been described as early as 1935,[14] its mechanism has been elusive so far.[15] In this study we present evidence that sulfinate adducts are not the immediate precursors leading to formation of the corresponding NADH analogues. The evidence suggests that sulfoxylate ion (HSO_2^-) is formed, which is postulated to be the active reducing species.



(4)

Experimental Section

Materials. NAD^+, 3-acetylpyridine-NAD^+, and thionicotinamide-NAD^+ were purchased from Boehringer, Mannheim, West Germany. 1-Methylnicotinamide and 10-methyl-5-deazaisoalloxazine were synthesized according to published procedures.[2a,16,17] Sodium dithionite was either from Baker Chemicals, Phillipsburg, N.J., or

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from Merck, Darmstadt, West Germany. Both batches had a dithionite content of 85-90% and they were used without further purification unless stated otherwise. All other chemicals and solvents were from commercial sources and they were used without further purification.

Formation of Sulfinate Adducts. NAD^+ (10^{-4} M) in the desired buffer was made anaerobic in a Thunberg-type cuvette by repeatedly evacuating and flushing with purified argon; the volume was usually 3 mL. An equimolar amount of dithionite was added by injecting 6 μL of a 5×10^{-2} M solution through the stopcock with a Hamilton syringe, equipped with a long needle. Formation of the adduct could be followed by the disappearance of dithionite absorption at 315 nm and the appearance of adduct absorption at the wavelength indicated in Table I.

For anaerobic stopped-flow measurements the respective solutions were prepared in tonometers, equipped with a side arm. Solutions were made anaerobic as described above and dithionite was then dissolved anaerobically. The desired concentrations of dithionite were obtained by weighing in solid dithionite into the side arm of the tonometer. After the stopped-flow spectrophotometer had been flushed with anaerobic buffer, the tonometers containing the anaerobic solutions were used directly on the instrument as reservoir syringes. The actual dithionite concentration was then checked by measuring the absorbance at 370-390 nm in the stopped-flow spectrophotometer before and after each experiment. On a weight basis the batch from Baker Chemicals had twice the absorbance of the batch from Merck (at 370-390 nm), although the observed rates of sulfinate adduct formation were identical within experimental error.

For the experiments in the presence of sulfite, the desired concentration was obtained by adding an equivalent from a sulfite stock solution to the buffer in the tonometer prior to anaerobiosis. After anaerobiosis dithionite was added from the side arm as described above.

Protonation of Sulfinate Adducts. NAD^+ analogue (10^{-4} M) at pH 11.7 (in 2 mM NaOH) was mixed with an equimolar amount of dithionite in a tonometer. Completion of sulfinate adduct formation was followed spectrophotometrically via a cuvette attached to the side arm of of the tonometer. This solution was mixed anaerobically with anaerobic acetate buffer (0.5 M, pH 4.7). The pH after mixing was 4.7. Trapping of sulfinate ion with formaldehyde was achieved by adding 10^{-2} M formaldehyde to the acetate buffer and subsequent mixing with a 10^{-4} M solution of sulfinate adduct at pH 11.7. With NAD^+ analogues I and III disappearance of sulfinate adduct was followed at 360 nm, while with V the appearance of oxidized 5-deazaflavin was followed at 390 nm.

Formation of 3-Acetylpyridine-NADH from NAD Sulfinate. NAD sulfinate (10^{-4} M) was prepared at pH 11.7 in an anaerobic cell and was mixed with an equal volume of anaerobic 0.5 M acetate (pH 5.0) containing 10^{-4} M 3-acetylpyridine-NAD^+. After mixing, electronic spectra were taken at regular time intervals until formation of 3-acetylpyridine-NADH was complete.

Purification of Commercial Dithionite. In a tonometer 30 mL of 0.1 M Tris/HCl buffer (pH 10.4) was made anaerobic as described above. The side arm contained $BaCl_2$ (0.01 mol) and dithionite (1 mmol). Upon mixing a white precipitate formed immediately and the solution was allowed to sit overnight. The supernatant was withdrawn anaerobically with a Hamilton syringe and used as a stock solution for further experiments.

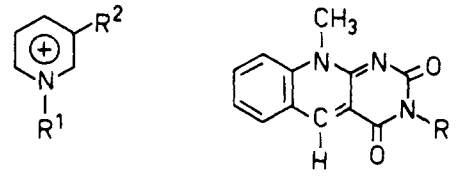
Results

Formation of Sulfinate Adducts. Three parameters were considered as important in studying stability and spectral properties of NAD^+ analogue sulfinate adducts: (1) dependence on changes in redox potential; (2) dependence on changes in acid-base properties; (3) the possibility of observing individual chromophores directly in the stopped-flow spectrophotometer. Changes in redox potential and acid-base properties will lead to changes in rates and equilibria of the individual reaction steps, thus assisting in the determination of the nature of the active species involved. Although the structure of 5-deazaflavins might lead to the impression that they are flavin analogues, their chemistry in many respects is that of a nicotinamide analogue.[2a,25] In our study 5-deazaflavins should be particularly useful because the oxidized chromophore absorbs at longer wavelengths than the reduced chromophore, in contrast to nicotinamides, where the reverse is true. Thus formation and disappearance of the oxidized chromophore can be directly observed spectrophotometrically with analogues V and VI, while formation and disappearance of the reduced chromophore can be directly observed with analogues I-IV.

Table I. Electronic Spectra of NADH Analogues and the Respective Sulfite as Well as Sulfinate Complexes[a]

analogue	sulfite adduct λ_{max}, nm	sulfite adduct ϵ, mM	1,4-dihydro λ_{max}, nm	1,4-dihydro ϵ mM	sulfinate adduct λ_{max}, nm	sulfinate adduct ϵ, mM
I	340	4.0	363	9.1	382	4.0
II	365	3.6	395	11.3	415	5.3
III	320	3.1	335	6.2	353	3.4
IV	314	3.3	360	6.2	372	3.2
V	310	10.6	316	12.0	314	6.4
VI	300	9.4	316	11.5	314	6.2

[a] Analogue IV was measured at pH 13 (0.1 M NaOH), all other analogues at pH 8.5 (0.1 M pyrophosphate).



(I-IV) (V, VI)

I R' = Rib-P-P-Rib-Ade
R² = -CO-CH₃
II R' = Rib-P-P-Rib-Ade
R² = -CS-NH₂
III R' = Rib-P-P-Rib-Ade
R² = -CO-NH₂
IV R' = CH₃
R² = -CO-NH₂
V R = H
VI R = $(-CH_2)_2-SO_3^-$

The electronic spectra of sulfinate adducts with I-IV characteristically show a red shift relative to the corresponding 1,4-dihydro derivatives (Figure 1, Table I). The extinction coefficients are always about half those of the corresponding 1,4-dihydro derivatives. The energy of the long-wavelength transition is not linearly correlated to the redox potentials of the corresponding NAD^+ analogues. Hence, it is unlikely to arise from a charge-transfer interaction between sulfinate ion and pyridinium ion coupled noncovalently in an ion-pair complex. With NAD^+ analogues I-III sulfinate adduct formation can be measured at 10^{-4} M concentrations of both nicotinamide and dithionite at pH 8.5. With the low-potential analogue IV, however, rapid hydration across the 5,6 double bond of the sulfinate adduct takes place. Hydration can be prevented when sulfinate adduct formation is measured at pH 13. Sulfinate adduct formation with 5-deazaflavins parallels that of the nicotinamides; however, the adducts are dianions at pH >8 due to deprotonation at N(1). At pH 13 V is present as the trianion due to additional deportonation at N(3). For this species K_d is 2×10^{-5} M, while K_d values for nicotinamides I-III can only be estimated. Since adduct formation is quantitative even at 2×10^{-5} M concentrations of both NAD^+ analogue and dithionite K_d must be $<10^{-6}$ M.

The kinetics of sulfinate adduct formation can be followed with the stopped-flow spectrophotometer and shows saturation with increasing dithionite concentration (Figure 2). With V and VI disappearance of the oxidized chromophore becomes biphasic at high dithionite concentrations (see Appendix).

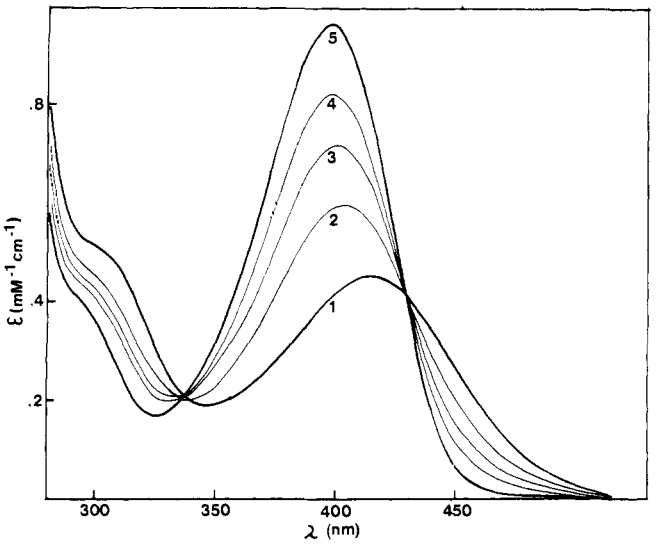


Figure 1. Anaerobic conversion of sulfinate adduct II (10^{-4} M, 0.1 M pyrophosphate, pH 8.5) to the 1,4-dihydronicotinamide: 1, sulfinate adduct of II; 2, 3, 4, 5, after 50, 90, 140, and 260 min, respectively.

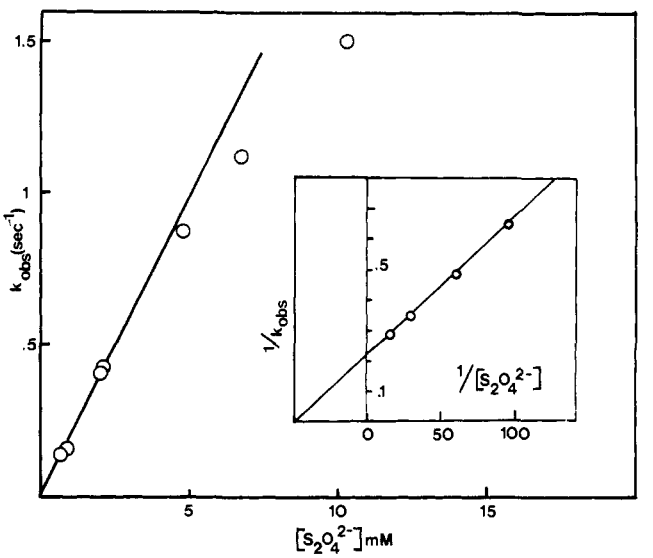


Figure 2. Saturation kinetics of sulfinate adduct formation: VI (10^{-4} M, 0.1 M pyrophosphate, pH 8.5) was mixed anaerobically with commercial dithionite in 0.1 M pyrophosphate (pH 8.5).

Table II. Rate Constants k_3 for Sulfinate Adduct Formation from NAD^+ Analogues and Dithionite

NAD^+ analogue	k_3, M^{-1} s^{-1}	E_0', mV
I	1050	−260
II	150	−290
III	47	−320
IV	1	−406
V	373	−280
VI	195	−305

Competition by sulfite, which is known to be the major contaminant of commercial dithionite preparations, was a likely possibility for the observed complications. Kinetic treatment for such a possibility is based on the following general mechanistic scheme:

$$A + B \underset{k_2}{\overset{k_1}{\rightleftharpoons}} C$$

$$A + D \xrightarrow{k_3} E$$

A represents the NAD^+ analogue, B represents the sulfite ion, C represents the NAD^+-sulfite complex, D represents dithionite, and E represents the sulfinate complex, respectively. A rigorous solution for the disappearance of A is given in the Appendix. It has been tested experimentally for analogue VI by studying the disappearance of A when varying the concentration of B and D (see Appendix).

The value for k_3 obtained from these studies agrees well with that obtained from the initial, linear part of a rate vs. dithionite relationship as in Figure 2.

Thus we can demonstrate that the rate of sulfinate adduct formation actually increases linearly with dithionite concentration. Furthermore, a linear free energy relationship is obtained when the redox potentials of I–IV are plotted vs. log k_3 (Figure 3), showing that the rate of sulfinate adduct formation is increasing with increasing (higher) potentials (Table II).

Removal of Sulfite Impurities. Since the kinetic results suggested the presence of significant sulfite impurities in our dithionite solutions we tried to remove sulfite as the barium salt, a method already suggested by Yarmolinsky and Colowick.[10] When a 3×10^{-2} M solution of dithionite at pH 10.4 is treated with 3×10^{-1} M barium chloride, barium sulfite

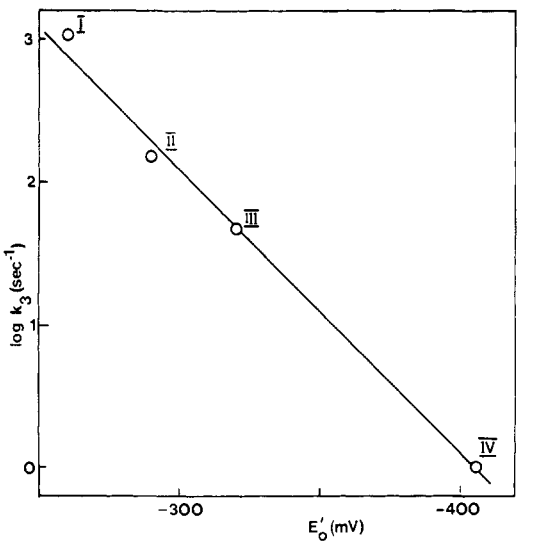


Figure 3. Linear free energy relationship for sulfinate adduct formation of nicotinamide analogues I–IV.

precipitates. The dithionite concentration remaining in the supernatant is 3×10^{-3} M as judged from the absorbance at 315 nm. This demonstrates that removal of sulfite drives dithionite dismutation rather than just eliminating the "impurities" present originally.

Decay of Sulfinate Adducts. At pH >11 sulfinate adducts of NAD^+ analogues are not converted to NADH analogues, indicating that the adduct deprotonated at the sulfinate function is inactive. By lowering the pH to 8.5, slow conversion to 1,4-dihydronicotinamide of the then partially protonated adduct is observed (Figure 1). As shown in Table III the electron-deficient nicotinamide analogue I reacts more slowly than the less electron-deficient analogues II and III. Since only the free sulfinic acid can be a presursor of dihydronicotinamide its pK must be one of the factors governing the pH dependence of product formation. To eliminate this complication we designed pH-jump stopped-flow experiments such that sulfinate adduct was completely protonated after the pH jump. It was prepared anaerobically in 2 mM NaOH (pH 11.7) from 10^{-4} M concentrations of both NAD^+ analogue and dithionite. At

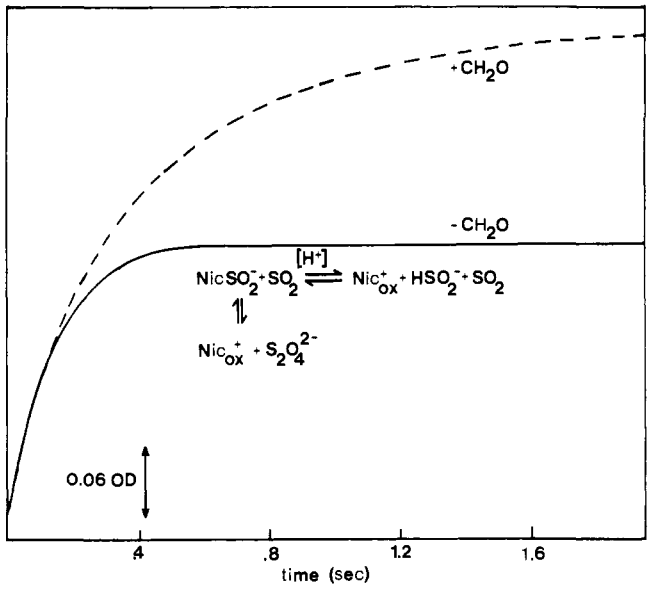


Figure 4. (—) Formation of oxidized nicotinamide analogue V at 390 nm after mixing sulfinate adduct (0.1 mM in 2 mM NaOH) with sulfate buffer (0.5 M, pH 1.5); (---) kinetic trace obtained when 10 mM CH_2O had been added to sulfate buffer.

Table III. Relative Rates of NADH Analogue Formation at 8.5 from 10^{-3} M NAD^+ Sulfinate

analogue	obsd first-order rate constant, s^{-1}
I	7×10^{-5}
II	1.78×10^{-4}
III	6.42×10^{-4}
IV	hydration

this concentration sulfite competition is negligible. The adduct was then mixed with anaerobic buffer (0.5 M acetate, pH 4.7), leading to complete protonation of the sulfinate (pK = 7.5) to give the sulfinic acid adduct.

With the analogues I and II disappearance of the adduct is observed initially, while with analogue V a corresponding appearance of the oxidized chromophore can be demonstrated (Figures 4 and 5). For V 67% of the total concentration is present as the free oxidized chromophore, while 33% is present as the sulfinic acid adduct. The rate of dissociation increases with decreasing potential; thus the first-order rate constant for analogue I is 0.38 s^{-1}, while that for analogue III is 4.62 s^{-1}. The corresponding first-order rate constant for analogue V is 3.08 s^{-1}.

These data clearly suggest that after the pH jump a new equilibrium is established between sulfinic acid adduct, oxidized nicotinamide, and an anionic species which has to be at the oxidation level of sulfoxylate. Most probably the sulfur dihydroxide tautomer $HOSO^-$ is formed initially. After the new equilibrium has been established, formation of dihydronicotinamide is observed in a much slower reaction (Figure 5). Formation of NADH is complete within 20 min.

The yield, as determined spectrophotometrically from the solution in the stop syringe, was 50% judged by the absorbance at 350 nm. NADH hydrate formation, although significant, is slower than NADH formation. Yield of hydrate was judged by the absorbance at 295 nm to be 5%. The relatively small yield at pH 4.7 must also be due to decomposition of dithionite, which is known to be unstable at acid pH.

The kinetics of NADH formation shows a bump at about 100 s after mixing. Presently we have no clear-cut interpretation for this kinetics. If one considers the complex sequence of events following the pH jump, however, simple kinetics are not to be expected (Scheme I).

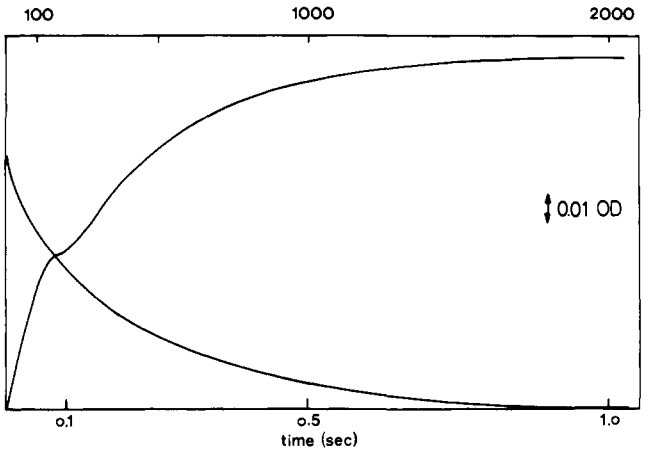


Figure 5. Decay of sulfinic acid adduct of NAD^+ at 350 nm (time scale on bottom) after mixing sulfinate adduct (0.1 mM in 2 mM NaOH) with acetate buffer (0.5 M, pH 4.7). Subsequent NADH formation is demonstrated by the increase of absorbance at 350 nm (time scale on top).

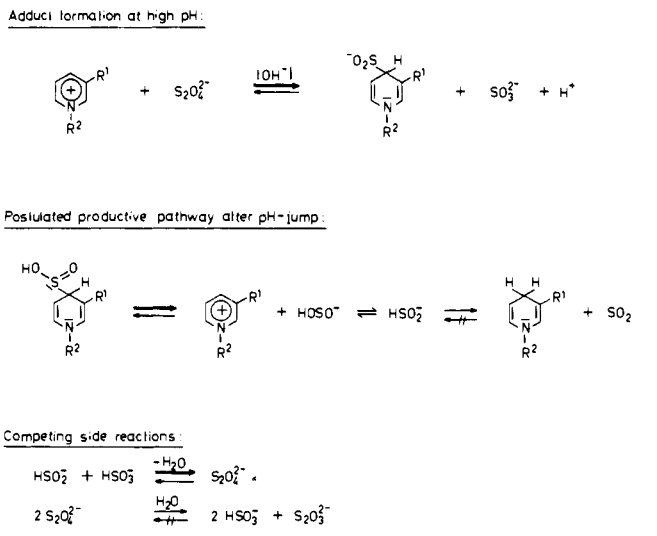
If the mechanism presented in Scheme I is valid, NADH formation should be prevented in the presence of formaldehyde, a known trapping reagent for sulfoxylate. This has been found to be the case with all analogues investigated. As shown in Figure 4 quantitative formation of oxidized chromophore can be demonstrated with V using 10^{-2} M CH_2O. In addition to the fast initial phase of 3.08 s^{-1} corresponding to the reaction in the absence of formaldehyde, a second slower phase (k = 0.91 s^{-1}) is observed, which should reflect the rate of sulfoxylate trapping by formaldehyde. The second-order rate constant thus obtained is 1.82×10^2 M^{-1} s^{-1}.

More importantly, our proposed mechanism predicts that a high-potential NAD^+ analogue should be reduced when mixed with the sulfinate adduct of NAD^+ at pH 5. Formation of NADH should only be partially observed. This has indeed been confirmed by the experiment: using 3-acetylpyridine-NAD^+ as the high-potential NAD^+ analogue formation of 3-acetylpyridine-NADH was almost quantitative (89%) while no NADH was detected spectrophotometrically (formation <1%).

Discussion

In view of the recent suggestions that nicotinamide in both biological and model systems might undergo reversible $1e^-$ oxidoreduction, the present study was undertaken with the aim

Scheme I



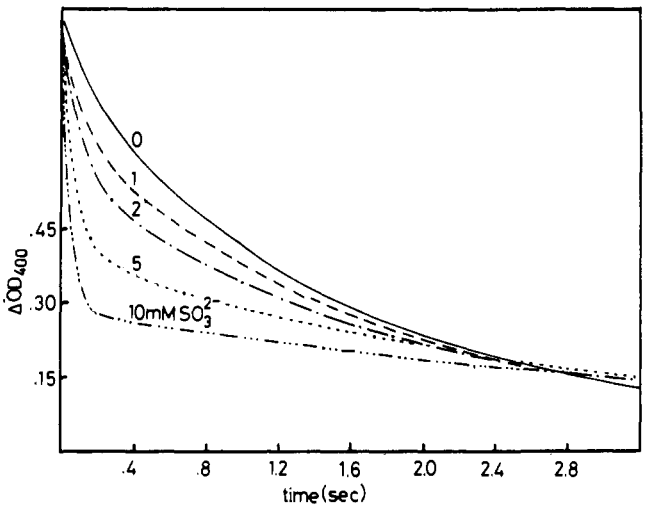


Figure 6. Disappearance of V1 at 400 nm (0.05 mM V1, 0.1 M pyrophosphate, pH 8.5) in the presence of dithionite (5 mM) and varying concentrations of sulfite.

to characterize the interaction of nicotinamides with an ambivalent $2e^-/1e^-$ reductant. Dithionite was the logical choice because it had been shown to undergo $1e^-$ oxidoreduction with both model flavins and flavodoxins, indicated by the linear dependence of the reduction rate on the square root of dithionite concentration.[8,9] This suggested that sulfinate radical, the product of homolytic dissociation of dithionite, was in fact the reactive species. Although Yarmolinsky and Colowick and others[10,12,13] have shown that nicotinamides form sulfinate adducts, which should arise from heterolysis of dithionite, no detailed information as to the kinetics of formation and decay of these adducts has been published to our knowledge.

We have now shown that formation rates of sulfinate adducts increase linearly with dithionite concentration; however, sulfite impurities present in commercial dithionite preparations lead to saturation kinetics. Since precipitation of sulfite as the barium salt does not lead to "purer" dithionite but rather induces dismutation it is clear that dithionite solutions at alkaline pH not only contain equilibrium amounts of sulfinate radicals. They also contain sulfinate ion and sulfite ion.

$$HSO_3^- + SO_2^{2-} \overset{(OH^-)}{\rightleftharpoons} S_2O_4^{2-} \rightleftharpoons 2\dot{S}O_2^- \qquad (5)$$

The equilibrium concentraton of these latter species as judged from our experiments appears to be very small. Dismutation can be induced, however, by either precipitating sulfite as the barium salt[20] or by complexing sulfoxylate at high pH with NAD^+ analogues, to form sulfinate adducts.

The probable presence of both sulfite and sulfinate ion has actually been mentioned several times in the early literature;[21-24] however, no equilibrium constants have been determined or estimated. In view of these facts it is not surprising that rate constants for sulfite addition to NAD^+ analogues, determined in the presence of dithionite, do not agree with those determined with sulfite alone.

The high thermodynamic stability of nicotinamide-sulfinate adducts relative to that of the corresponding cyanide or sulfite adducts is a consequence of very low off-rates. The on-rates differ no more than tenfold for the respective adducts while the off-rates differ by more than a factor of 10^3. This stabilization is probably a consequence of homoconjugation of the lone pair at sulfinate sulfur with the heteroaromatic π electrons.

Mechanism. The mechanism proposed in Scheme I, namely, that sulfinate ion (HSO_2^-) is the precursor of NADH analogue formation, is based on the following arguments and evidence.

1. If sulfinate adduct would be directly converted to 1,4-dihydronicotinamide with elimination of SO_2, the deprotonated and not the protonated adduct should be active. The evidence shows, however, that the deprotonated form is inactive.

2. Increasing rates of dissociation of sulfinic acid adducts with increasing electron density in the nicotinamide (decreasing redox potential) indicate that an anionic species is eliminated. Heterolytic dissociation can only lead to oxidized nicotinamide and sulfoxylate ion.

3. 3-Acetylpyridine-NAD^+, when added at pH 5 to NAD sulfinate, is converted with the same rate and yield to 3-acetylpyridine-NADH as 3-acetylpyridine-NAD sulfinate at pH 5 (Blankenhorn and Zoephel, unpublished results).

4. The sulfur dihydroxide tautomer of sulfoxylate, $HOSO^-$, cannot be the immediate donor leading to formation of 1,4-dihydronicotinamide because it is an oxidant rather than a reductant.[21] The sulfinate tautomer HSO_2^-, however, should be a good hydride donor in view of the fact that SO_2 is a good leaving group.

The rate of 1,4-dihydronicotinamide formation will then depend primarily on the actual concentration of HSO_2^- ion and the acceptor properties of the nicotinamide analogue. While increasing nicotinamide potential will tend to decrease the HSO_2^- ion concentration because of higher adduct stability, the actual hydride transfer step should increase in rate. Hence, it is difficult to predict how formation rates of 1,4-dihydronicotinamide should depend on substituent effects.

A further complication of the kinetics is to be expected from dihydronicotinamide hydration, particularly for low-potential analogues. Finally, dithionite itself is acid labile, leading to formation of sulfite and thiosulfate (Scheme I). A detailed kinetic investigation of these problems is presently underway in our laboratory.

To obtain high yields of NADH analogues the competing side reactions must either be reversible (NADH formation is essentially irreversible) or slow.

For each NAD^+ analogue there will be a pH optimum for product formation such that adduct stability is already small (adducts are not productive) and dihydronicotinamide hydration is still slow. In case of very low adduct stability the concentration of HSO_2^- is determined by the dithionite dismutation equilibrium. Protonation of HSO_2^- leads to its decomposition while reaction with NAD^+ analogues produces NADH analogues.

The mechanism proposed for dithionite-dependent reduction of NAD^+ analogues reflects the important differences in the redox (bio)chemistry of flavins and nicotinamides. Flavins are characterized by their relatively high thermodynamic radical stability and they react with sulfinate radical by a $1e^-$-transfer mechanism. Nicontinamides, however, with their very low thermodynamic radical stability,[2a] do not react with sulfinate radicals. A second thermodynamic factor, the high stability of adducts with nucleophiles, is characteristic for nicotinamides, while the corresponding adducts of flavins are thermodynamically unstable.[25]

5-Deazaflavins, in spite of their flavin-like structure, have to be considered as nicotinamide analogues as far as radical stability and adduct stability are concerned.[25] This has again been demonstrated in the present study. In one type of reaction, however, 5-deazaflavins must be considered as low-potential flavin analogues rather than nicotinamide analogues: redox transfer between flavins and nicotinamides characterized by charge-transfer complexes as probable intermediates.[2a,4] If one accepts that $1e^-$ transfer is thermodynamically blocked and covalent catalysis is not evident, this latter reaction has to be characterized as a hydride transfer also.

Appendix

The results shown in Figure 2 suggest that sulfite impurities

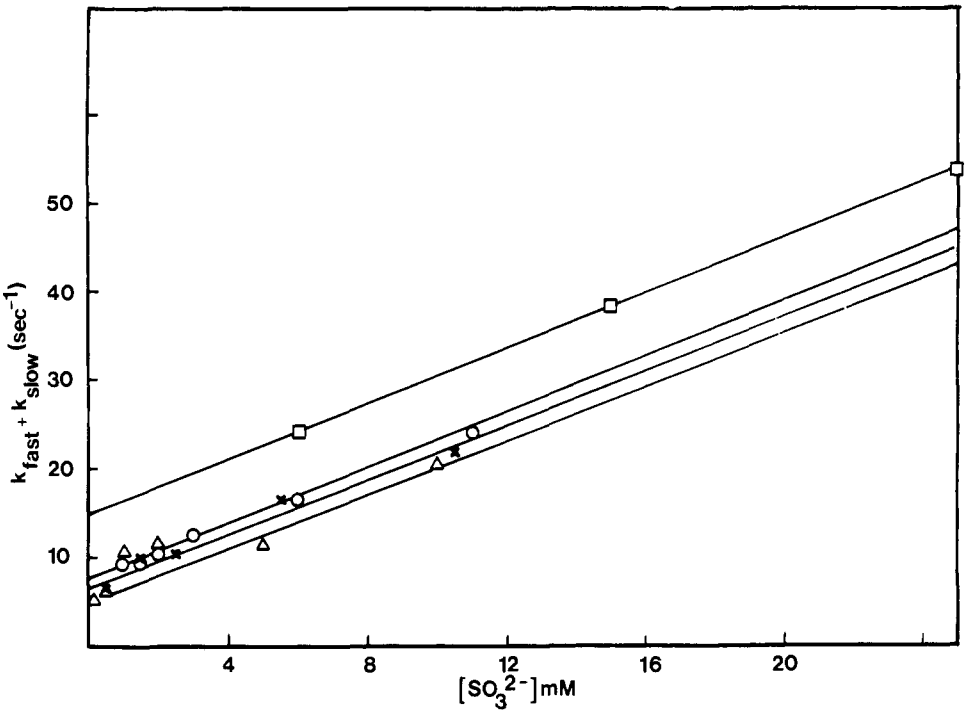


Figure 7. Plot of $k_{fast} + k_{slow}$ for different dithionite concentrations when varying sulfite. Conditions as above; △, ×, ○, □ = 1, 5, 10, and 50 mM, respectively.

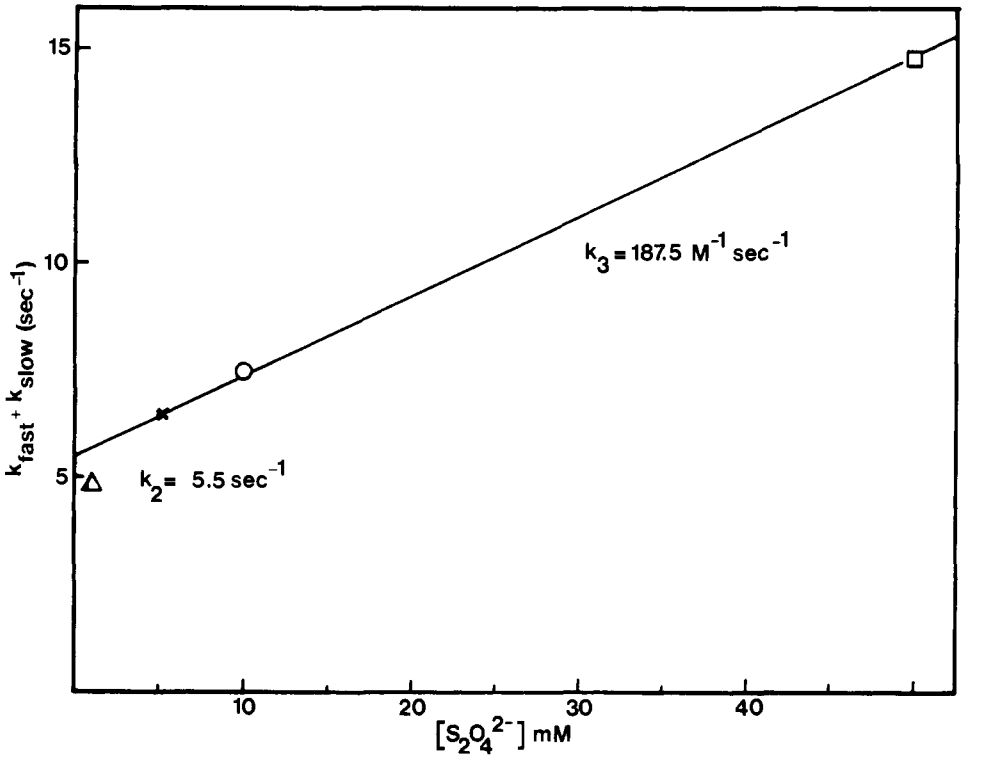


Figure 8. Replot of the intercepts obtained in Figure 7 vs. dithionite giving the second-order rate constant k_3 for sulfinate adduct formation of V1.

in dithionite compete with dithionite. This is evidenced at high concentrations of dithionite by the deviation from purely second-order behavior and by the increasing proportion of biphasic nature of the kinetic traces. Exogenous addition of sulfite ion to the dithionite solution accentuates the kinetic phenomenon (Figure 6).

The kinetics can be described by a reversible equilibrium binding of sulfite ion to NAD^+ analogue illustrated by the following scheme:

$$\begin{aligned} A + B &\underset{k_2}{\overset{k_1}{\rightleftharpoons}} C \\ A + D &\underset{k_4}{\overset{k_3}{\rightleftharpoons}} E \end{aligned} \qquad \text{(A1)}$$

where A is the NAD^+ analogue, B is sulfite ion, C is the sulfite complex, D is dithionite, and E is the sulfinate complex and subsequent products.

A rigorous analytical solution to the above scheme can be

derived by algebraic treatment of the rate equations

$$-d[A]/dt = k_1[A][B] + k_3[A][D] - k_2[C] - k_4[E]$$
$$-d[C]/dt = k_2[C] - k_1[A][B]$$
$$-d[E]dt = k_4[E] - k_3[A][D] \quad (A2)$$

as described by Frost and Pearson[18] assuming the concentrations of B and D to be in large excess of A. Solution of the rate equations first involves a series of secular equations in λ (eq A3), where λ will be a particular combination of rate constants which describe the exponent of a given exponential phase.

$$\begin{vmatrix} k_1[B] + k_3[D] - \lambda & -k_2 & -k_4 \\ -k_1[B] & k_2 - \lambda & 0 \\ -k_3[D] & 0 & k_4 - \lambda \end{vmatrix} = 0 \quad (A3)$$

Solving eq A3 for λ gives three solutions:

$$\lambda_1 = 0$$
$$\lambda_2 = \tfrac{1}{2}(\alpha + \beta) \quad \alpha = k_1[B] + k_3[D] + k_2 + k_4$$
$$\lambda_3 = \tfrac{1}{2}(\alpha - \beta) \quad \beta = \alpha^2 - 4[k_1k_4[B] + k_2k_3[D] + k_2k_4] \quad (A4)$$

Substitution of the expressions for λ and solving for relative constants results in a general solution for the concentrations of A, C, and E during the course of the reaction:

$$[A] = \sum_r Q_r \exp(-\lambda_r t)$$
$$[C] = \sum_r Q_r \left(\frac{k_1[B]}{k_2 - \lambda_r}\right) \exp(-\lambda_r t) \quad (A5)$$
$$[E] = \sum_r Q_r \left(\frac{k_3[D]}{k_4 - \lambda_r}\right) \exp(-\lambda_r t)$$

A specific solution for the Q_r coefficients can be obtained by assuming the conditions at time $t = 0$, that the total NAD^+ analogue concentration is present as A and that [C] = [E] = 0. Substituting these constraints into eq A5 and solving for values of Q_r yields an analytical solution for the competitive scheme (eq A1):

$$[A] = [A^0]\left[\frac{k_2k_4}{\lambda_2\lambda_3} + \frac{(k_2 - \lambda_2)(k_4 - \lambda_2)}{\lambda_2(\lambda_2 - \lambda_3)} \times \exp(-\lambda_2 t) + \frac{(k_2 - \lambda_3)(k_4 - \lambda_3)}{\lambda_3(\lambda_3 - \lambda_2)} \exp(-\lambda_3 t)\right] \quad (A6a)$$

$$[C] = [A^0]\left[\frac{k_2k_4k_1[B]}{\lambda_2\lambda_3k_2} + \frac{k_1[B](k_4 - \lambda_2)}{\lambda_2(\lambda_2 - \lambda_3)} \times \exp(-\lambda_2 t) + \frac{(k_4 - \lambda_3)k_1[B]}{\lambda_3(\lambda_3 - \lambda_2)} \exp(-\lambda_3 t)\right] \quad (A6b)$$

$$[E] = [A^0]\left[\frac{k_2k_3[D]}{\lambda_2\lambda_3} + \frac{(k_2 - \lambda_2)k_3[D]}{\lambda_2(\lambda_2 - \lambda_3)} \times \exp(-\lambda_2 t) + \frac{(k_2 - \lambda_3)k_3[D]}{\lambda_3(\lambda_3 - \lambda_2)} \exp(-\lambda_3 t)\right] \quad (A6c)$$

The measurements in the text of this paper follow the disappearance of species A (which absorbs light) to species C and E, neither of which absorbs light at the wavelengths of choice. Consequently, the kinetics are described by eq A6a.

The kinetics of NAD^+ analogue (V1) reduction in the presence of sulfite ion are biphasic with no apparent nonexponential (eq A6a). This would be observed if k_2, k_4, or both were nearly zero. Curve fitting eq A6a by nonlinear least-squares methods,[26] varying k_1, k_2, k_3, and k_4, to the kinetic traces indicated that k_4 was nearly zero. This can be visualized directly from the sum and difference of λ_2 (observed fast rate) and λ_3 (observed slow rate):

$$k_{fast} + k_{slow} = \lambda_2 + \lambda_3 = k_1[B] + k_2 + k_3[D] + k_4 \quad (A7a)$$
$$k_{fast} - k_{slow} = \lambda_2 - \lambda_3 = [(k_1[B])^2 + (k_3[D])^2 + k_2^2 + k_4^2 + 2k_1[B]k_3[D] + 2k_2(k_1[B] - k_3[D]) - 2k_4(k_1[B] - k_3[D]) - 2k_2k_4]^{1/2} \quad (A7b)$$

Figure 7 shows a plot of $k_{fast} + k_{slow}$ according to eq A7a for V1. The slope of the lines give the value of k_1 of 1590 M^{-1} s^{-1}. A replot of the intercepts (Figure 8) gives a value of k_3 of 187.5 M^{-1} s^{-1} from the slope of the line and a value of k_2 or k_4 of 5.5 s^{-1}.

The value obtained for k_3 agrees well with that obtained from the initial slope of k_{obsd} vs. dithionite relationship (187.5 vs. 195 M^{-1} s^{-1}). It appears as very probable, therefore, that the observed saturation kinetics are indeed a result of sulfite competition.

References and Notes

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